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LETTER

Cloning and Functional Analysis of cDNAs with Open Reading Frames for 300 Previously Undefined Genes Expressed in CD34+ Hematopoietic Stem/Progenitor Cells

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ABSTRACT

Three hundred cDNAs containing putatively entire open reading frames (ORFs) for previously undefined genes were obtained from CD34+ hematopoietic stem/progenitor cells (HSPCs), based on EST cataloging, clone sequencing, in silico cloning, and rapid amplification of cDNA ends (RACE). The cDNA sizes ranged from 360 to 3496 bp and their ORFs coded for peptides of 58-752 amino acids. Public database search indicated that 225 cDNAs exhibited sequence similarities to genes identified across a variety of species. Homology analysis led to the recognition of 50 basic structural motifs/domains among these cDNAs. Genomic exon-intron organization could be established in 243 genes by integration of cDNA data with genome sequence information. Interestingly, a new gene named as HSPC070 on 3p was found to share a sequence of 105bp in 3' UTR with *RAF* gene in reversed transcription orientation. Chromosomal localizations were obtained using electronic mapping for 192 genes and with radiation hybrid (RH) for 38 genes. Macroarray technique was applied to screen the gene expression patterns in five hematopoietic cell lines (NB4, HL60, U937, K562, and Jurkat) and a number of genes with differential expression were found. The resource work has provided a wide range of

information useful not only for expression genomics and annotation of genomic DNA sequence, but also for further research on the function of genes involved in hematopoietic development and differentiation.

[The sequence data described in this paper have been submitted to the GenBank data library under the accession nos. listed in Table 1, pp 1548-1552.]

INTRODUCTION

TOP
ABSTRACT
INTRODUCTION
RESULTS
Methods
REFERENCES

The Human Genome Project now is at a historic turning point, from genomic DNA sequencing to functional genomics. According to the announcement from both public domain and private sector sequencing efforts, a "working draft" of the human genome sequence was just obtained, and the completion of the sequence will be achieved before the end of 2001 (Collins et al. 1998; Venter et al. 1998; Marshall 1999, 2000). The gene discovery and understanding of genetic information will require annotation of the sequence data using bioinformatic tools (Burge and Karlin 1997). Meanwhile, cloning of full-length cDNA has been listed as one of the major tasks of the current phase of genomic science (Collins et al. 1998). The integration of cDNA sequences with the genomic ones will greatly ease the identification of transcriptional units, as well as their mRNA levels and specificities in cells/tissues as a result of a fine regulation of the transcriptional expression at genomic level (Dunham et al. 1999; Hattori et al. 2000). Moreover, the cDNA project links directly to protein structural biology and exerts significant impact on the medical genetics and biotechnology/pharmaceutical industries.

Hematopoietic stem/progenitor cells (HSPCs) possess important roles for the physiological and pathological hematopoiesis, one of the essential areas in biomedicine, and the molecular basis of hematopoiesis remains to be better understood (Morrison et al. 1995, 1997). Over the last 3 years, we have been undertaking to catalog the expressed sequence tags (ESTs) from cDNA libraries of CD34+ HSPC populations from both umbilical cord blood (Mao et al. 1998) and adult bone marrow (Gu et al. 2000). This approach turned out to be very successful in terms of both gene expression profiling and discovery of novel genes in an efficient way. More recently, we have been extending this work to the cloning and sequencing of full-length cDNAs for previously undefined genes and to investigate their functions.

In this work, we report on the characterization of structural/functional features, chromosomal localization, and transcriptional expression patterns in different hematopoietic cell lines of 300 cDNAs with putatively entire open reading frames (ORFs) isolated from CD34+ cells. We also tried to integrate these data with the genomic sequence information and to propose some strategies to deal with the major challenges in expression genomics facing the completion of the human genomic sequences in the coming 1 or 2 years.

RESULTS

▲ TOP
▲ ABSTRACT
▲ INTRODUCTION
• RESULTS
▼ Methods
▼ REFERENCES

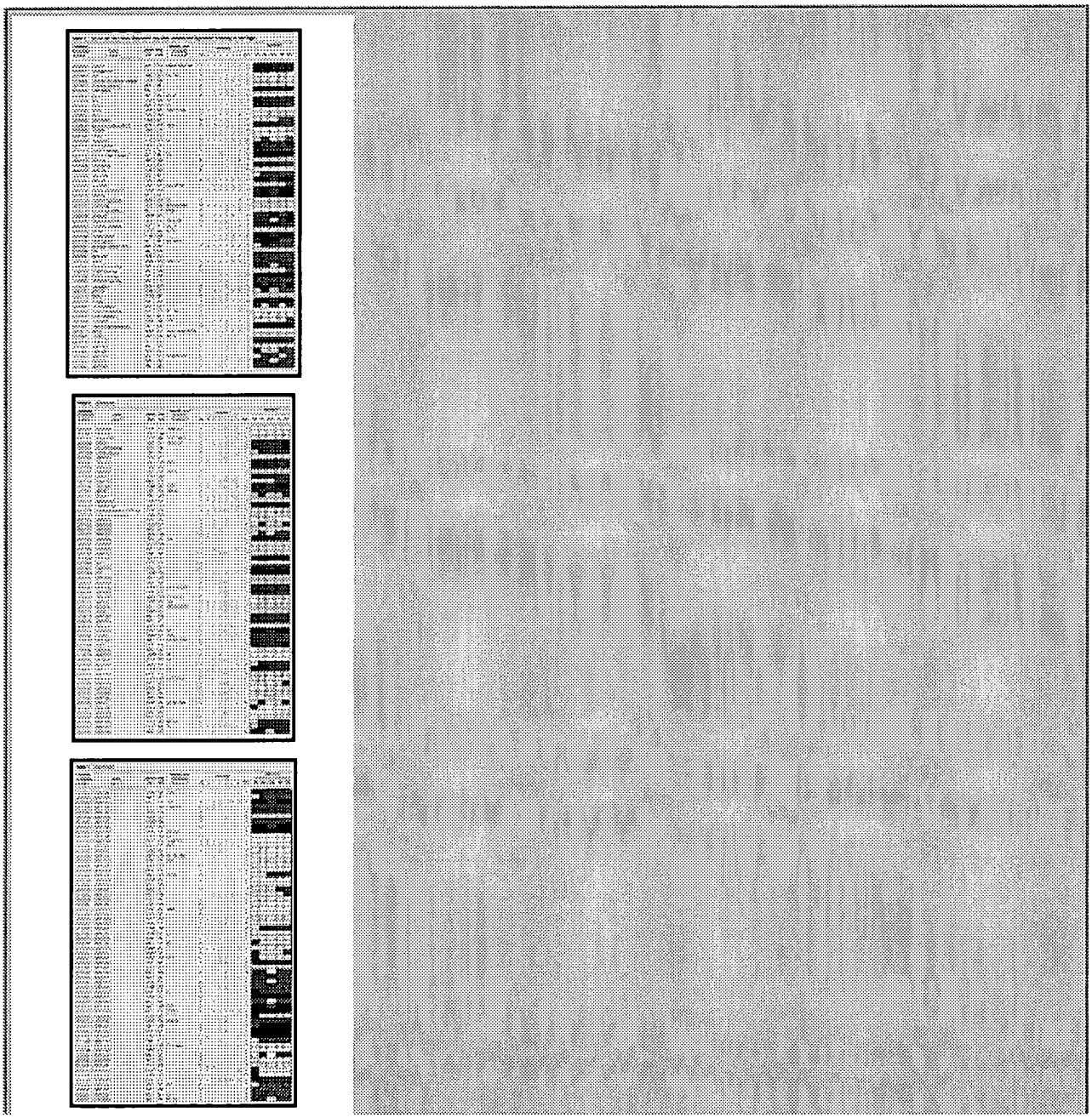
Primary Gene Expression Profiles of CD34+ HSPCs

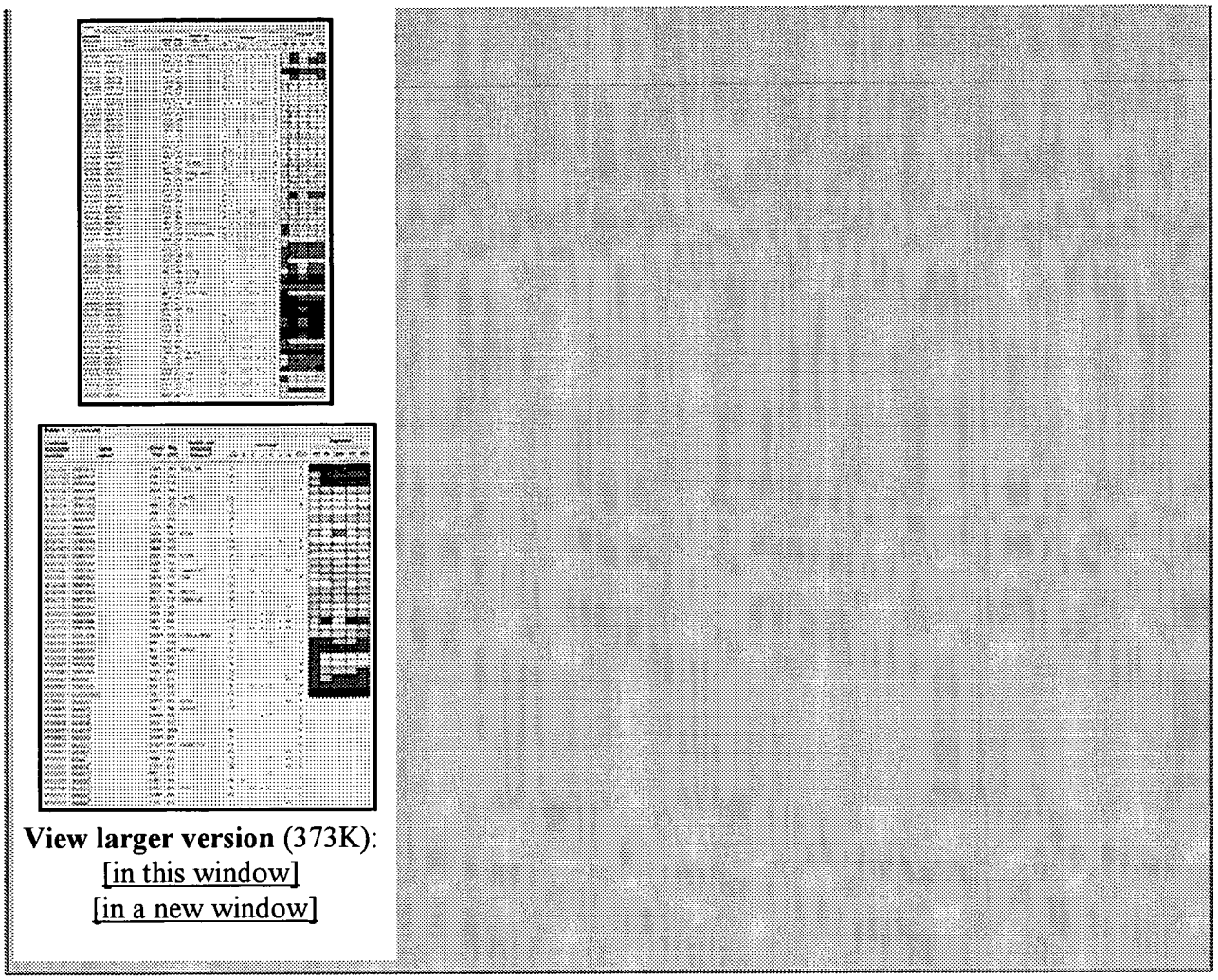
RT/PCR-based Capfinder cDNA libraries were constructed using mRNA from highly purified CD34+ HSPCs of cord blood and adult bone marrow, using methods described previously (Mao et al. 1998). In total, 1×10^6 recombinant clones were obtained from CD34+ cell library of cord blood origin (CB) and 0.5×10^6 clones were acquired from that of bone marrow (BM). The average size of inserts in both libraries was 1.2 kb. Among 9866 and 4142 EST sequences obtained from CB and BM CD34+ cell libraries, respectively, the repetitive DNA elements, rRNA, and mitochondrial DNA sequences accounted for 11.7% and 17.3% of the total, respectively. After eliminating these sequences, the meaningful ESTs were classified into known gene, dbEST, and novel EST groups by database search. For useful ESTs from both origins, the known and named gene groups occupied the largest portion (5377 out of 7376 from cord blood and 2265 out of 3424 from bone marrow, respectively); the list of all ESTs corresponding to known genes from both origins is now available at http://www.chgc.sh.cn. The ESTs representing undefined genes (dbEST and novel EST groups) were assembled into 2060 clusters, which then served as candidates for cloning of full-length coding sequences.

Cloning of cDNAs with Putatively Entire ORF for Previously Undefined Genes

Sequences of cDNA clones representing 2060 EST clusters of undefined genes were obtained. Those clones with continuous sequences encoding at least 100 amino acids (with an exception of a few smaller ORFs bearing very high homology to the known small genes) were checked for the presence of putatively entire ORFs using the following criteria. First, when a sequence had high homology to a known gene, its ORFs were compared with each other. If the amino acid sequences of both ORFs initiated by an ATG codon could be reasonably aligned, the ORF contained in the novel gene cDNA was defined as a putatively complete one. Second, those sequences without homology to known genes were searched for in-frame stop codons upstream of an ATG codon-initiated ORF of >100 amino acids. If no such stop codon was found ahead of an ORF, the nucleic acid sequence flanking the first ATG should bear similarity to the well-conserved KOZAK motif (Kozak 1986). The above analysis revealed that 222 of our clones might contain an entire ORF. In 78 EST clusters, an obvious but incomplete reading frame was present. Different methods were employed to prolong the ORF in these 78 clones until complete ones were considered to be reached according to the aforementioned criteria. In silico cloning with dbEST extension allowed us to obtain 69 putative entire ORFs, which were then confirmed by sequencing of material cDNA clones obtained by appropriately designed RT-PCR. Finally, for those sequences that could not be extended properly with an electronic approach, rapid amplification of cDNA ends (RACE) was applied to get the 5' or 3' ends with Marathon-ready cDNA libraries from appropriate tissue origins. Another nine entire ORFs were cloned and sequenced this way. In total, 300 cDNAs with putatively entire ORFs were obtained. Their nucleic acid sequences were 360-3496 bp in length and their ORFs coded for peptides of 58-752 amino acids. The major features of each gene are summarized in Table 1. It is worth pointing out that, although a 3' poly(A) sequence or a polyadenylation signal was found in most (214/300) cDNAs as evidence of containing the complete 3' UTR, the integrity of the 5' UTR could not be certain in the majority of the cDNAs.

In the remaining 1760 EST clusters corresponding to previously undefined genes, 512 clusters contained partial reading frames, 806 represented 3' UTRs as they had no obvious reading frames but presented polyadenylation signal and poly(A) tails, and the remaining 442 contained sequences of which the features should be further analyzed.





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Functional Significance Indicated by Homology Comparison with Genomic Sequences through Evolution

It is well accepted that homologous genes often share similarities at sequence and/or functional levels (Henikoff et al. 1997). Hence, sequence similarity acquisition is an efficient method to predict the function of a novel gene. Members belonging to the same gene families could be assumed/determined with this strategy and conserved genes often show conserved sequence elements within the important functional domains or motifs. Based on this consideration, putative ORFs from model organisms with completed genome sequence, including bacteria, *Saccharomyces cerevisiae, Caenorhabditis elegans*, and *Drosophila*, and ORFs of identified genes from *Arabidopsis* and mammals (excluding primates) were retrieved to compare the amino acid sequence similarities with those of ours (Table 1; Fig. 1). Sequences with similarity >25% over a region of 50-100 amino acids were considered here to have some homology (Russell et al. 1997). Among our 300 cDNA sequences, 21 share similarity to the coding sequences in all species examined, indicating that they are well-conserved genes and important for cell life. In fact, 16 of these 21 genes have assigned functions. A total of 204 cDNAs contained ORFs with >25% similarity to the sequences in at least one species. Functional clues have been available in 105 of these 204 genes. Taken as a whole, at least 225 genes identified in the current work are evolutionarily conserved. Interestingly, as shown in Figure 1, an increased gradient of similarity in terms of both number

of related genes and the degree of homology is present from bacteria to *Drosophila*. In the case of *Arabidopsis*, only part of the genomic sequence is available in the public database. However, 66 of our cDNAs found their homologs in this plant. As expected, the number of genes with high homology (>50%) was great in mammals. The fact that 75 cDNAs had so far no obvious similarity to any genes across different species implied that they might be functionally specific genes acquired relatively late during evolution.

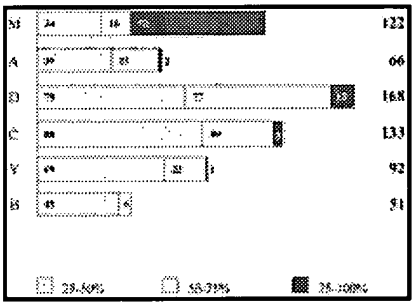

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Figure 1 Homology comparison of ORFs contained in our cDNAs to known genes from different model organisms. The horizonal blocks represent numbers of the ORFs bearing homology to genes in a given species; different colors indicate the degree of homology. Each number listed at *right* indicates the total number of our ORFs having homologous genes in that organism. (B) Bacteria; (Y) yeast; (C) *C. elegans*; (D) *Drosophila*; (A) *Arabidopsis*; (M) mammals not including primates.

Structural and Functional Assignment with Bioinformatic Prediction

Basic structural motifs predicted by some algorithms on the primary structure in the ORFs are listed in Tables 1 and 2, including leucine zipper, C2H2 zinc finger, and C3HC4 ring finger. Some consensus patterns of protein kinase, growth factor, and cytokine receptor-associated protein were also found by such methods. However, caution should be taken in interpreting these data. For instance, leucine zipper motif was predicted on primary structure in 12 ORFs using the Motifs software in the GCG package. Further analysis with Coilscan and Peptidestructure programs also provided by the GCG package revealed, nevertheless, that only 1 of these 12 leucine zippers was located in a coiled-coil structure. Because a typical leucine zipper should be included in a coiled-coil domain, this result indicates the importance of integration of information generated by different prediction methods, including those for conserved motifs at primary sequence level and those for secondary or higher structures. In analyzing the signal peptide, two different approaches, Spscan (in GCG package) and signalP (http://www.cbs.dtu.dk/services/Signalp/) were applied to our ORFs. The former algorithm is based on the weight matrix method in concert with McGeoch's discrimination of a minimum signal peptide, whereas the latter is based on two neural network methods for recognition of signal peptides and their cleavage sites. Of note, only cleavable signal peptides, but not the uncleavable ones like signal anchor and internal signal, can be detected with these algorithms. Interestingly, the two approaches gave quite coherent results in predicting putative amino-terminal potential signal peptides in 11 ORFs, including 8 with α-helix transmembrane domains outside the signal peptide region. One such example was an ORF with both signal peptide and 6-transmembrane domains (HABC7, GenBank accession no. AF038950), which contains an ABC transporter family signature. We therefore speculated this ORF encodes a putative transmembrane transporter protein.

View this table: [in this window] [in a new window]	**Table 2. List of the Abbreviations of Motifs and Structure Features in Table 1**

Genomic Organization and Alternative Splicing Identification

Of our genes, 243 were preliminarily characterized in terms of exon-intron organization after comparison of cDNA sequences with the genomic sequences in the database (Table 1). The estimated genomic sizes of these genes spanned 384 bp to 144 kb, containing 1 to >17 exons, and correspondingly 0 to >16 introns. The size distribution of the exons was from 20 bp to 2023 bp, whereas that of characterized introns ranged from 77 bp to 86 kb. Of note, 17 genes composed of only 1 exon varied in sizes from 384 bp (HSPC016, accession no. AF077202) to 1346 bp (P47, accession no. AF078856). On the other hand, cDNAs of short length could contain multiple exons. For example, HSPC245 (accession no. AF151079), consisting of 5 exons, and HSPC024 (accession no. AF083241), consisting of 7 exons, were only 497 bp and 581 bp in length, respectively.

During the characterization of the genome organization of our genes, some alternative splicings were determined. A 453-bp sequence in hSC2 (accession no. AF038958) was deleted in an isoform (accession no. AF038959), which was only found in CD34+ cells so far, whereas *LYPL-A1* (accession no. AF077198) used a 48-bp stretch that did not exist in the short form transcript (accession no. AF077199) (Fig. 2A). The fact that these alternatively used sequences are located in ORFs in an in-frame way supports the idea that these are physiologically existing isoforms and not artifacts in cDNA library construction. Indeed, the isoforms of the two genes were further confirmed by RT/PCR assay (data not shown). Interestingly, the cDNA sequence of *HSPC070* (accession no. AF161555) located on chromosome 3p25 was found to share a 105-bp stretch in the 3' UTR including the polyadenylation signal with that of RAF oncogene (accession no. X03484) (Bonner et al. 1986) in reversed orientation (Fig. 2B). This was further confirmed by the draft genome sequence from GenBank (AC018494, AC018500, AC026153, and AC026170) (see legend for Fig. 2B).

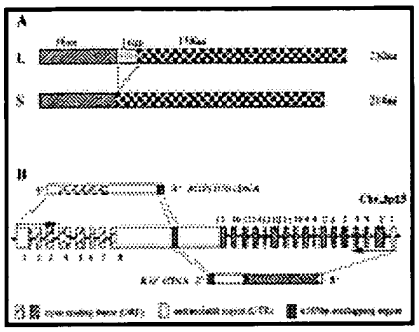

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Figure 2 (*A*) Alternative splicing present in lysophospholipase gene transcripts as long (L, accession no. AF077198) and short (S, accession no. AF077199) forms. The numbers indicate the amino acid positions of deduced proteins. Note that the ORF is maintained in the alternatively spliced S isoform. (*B*) Overlapping of HSPC070 (accession no. AF161555) and *RAF* genes located on opposite DNA strands at the same locus. Both genes are mapped to the same region on chromosome 3p25. The comparison of sequences between cDNAs and genomic DNA has allowed the exon-intron structure of both genes to be established, with exons represented by boxes and their numbers indicated. Note that a stretch of 105 bp is shared by the 3' UTRs of both genes. Arrows indicate the orientations of transcription.

Chromosomal Mapping

Chromosome localization is an important aspect of a gene's general information. Combining strategies of bioinformatics acquisition from both UniGene and other databases, and radiation hybrid (RH), a total of 230 genes were mapped to proper chromosome positions (Fig. 3). Among 55 genes mapped with G3 or GeneBridge 4 RH panels, 38 had not been mapped previously, whereas the remaining 20 RH results showed good concordance with those by electronic mapping. The detailed mapping results are available at http://www.chgc.sh.cn. Of note, the 5 C2H2 zinc finger genes are all located on chromosome 19.

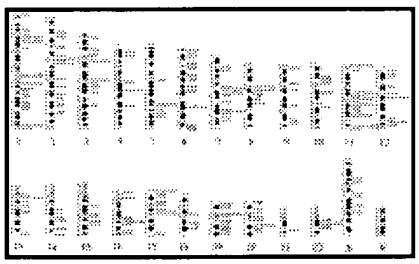

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Figure 3 Chromosome localization of 230 previously undefined genes by applying both STS searching and radiation hybrid (those marked with #). Detailed mapping information can be obtained from http://www.chgc.sh.cn

Expression Patterns in Different Tissues and in Distinct Hematopoietic Cell Lines

Among the 300 cDNAs, 270 could be analyzed using electronic Northern because their dbEST hits were available from UniGene resource. As shown in Table 1, most (207/270) genes showed ubiquitous transcriptional expression patterns as their corresponding ESTs were found in >10 tissues. The expression was found in a more selective way (<10 tissues) in 63. Only 13 showed relatively restricted expression in hematopoietic organs/tissues (bone marrow, foetal liver, spleen, lymph nodes, etc.). To explore the biological meanings of our genes in hematopoiesis, 285 cDNAs from the CB CD34+ cell library were also

examined using cDNA macroarray for their expression levels in hematopoietic cell lines (the array membrane used in this work did not include the 15 cDNAs from the BM CD34+ cell library). The cDNA probes were prepared with mRNAs isolated from NB4 (granulocytic), HL60 (granulocytic), U937 (monocytic), K562 (erythro-megakaryocytic), and Jurkat (T lymphocytic) cell lines representing distinct lineages of hematopoietic cells. The RNA quality was ensured with appropriate ratio between 18S and 28S rRNA bands on agarose gel electrophoresis, and the labeling efficiencies of cDNA probe were confirmed to be >50%. To evaluate the expression levels, the membranes were exposed to Phosphor screen and the relative intensity of each gene was quantified with FLA-300 detection system. Hybridization signals in separate experiments with different membranes and/or probes were calibrated using housekeeping genes including *GAPDH* and total amount of signals on the membrane as reference. The feasibility of the technology system was confirmed by reproducible results of the paralleled duplicate spots on the same membrane (Fig. 4A) and with independent tests on different membranes (Fig. 4B). The comparison of expression levels in different cell lines for 285 genes examined is shown on Table 1 (normalization with different references revealed similar results though only those based on *GAPDH* control are shown). Although most genes exhibited expression in all five cell lines, 35 of them displayed restricted expression in only one or two lineages. Northern blot analysis was performed for three genes, HSPC070, ZNF254, and HSPC135. According to the UniGene data, HSPC070 has a ubiquitous expression pattern, whereas the expression of ZNF254 and HSPC135 could be restricted to hematopoietic system (Table 1). Indeed, Northern blot analysis showed that HSPC070 was expressed in a variety of tissues (Fig. 4C) whereas no obvious transcriptional expression of ZNF254 and HSPC135 was detected in these tissues (data not shown). However, the three genes were all found expressed in most of the hematopoietic cell lines examined in this work.

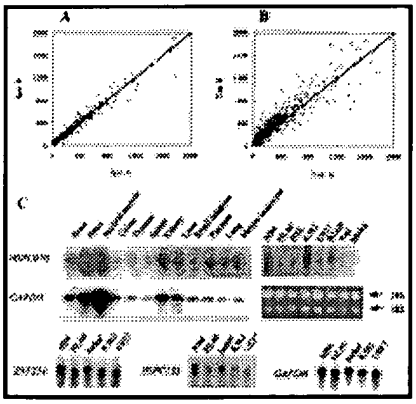

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Figure 4 Regression analysis of the cDNA array results (*A*,*B*) and Northern blot analysis of three cDNAs (*C*). (*A*) The scatterplot of detected signal intensity for duplicate spots on the same membrane. (*B*) Scatterplot of detected signal intensity for the corresponding dots in two membranes with independent tests from RNA of same cell origin. All signals are normalized by using *GAPDH* gene as internal control. The figures were made with Microsoft Excel spread sheet and the correlation line was indicated. (*C*) Northern blot analysis of *HSPC070*, *ZNF254*, and *HSPC135*. (*Top*) *HSPC070* with a ubiquitous tissue expression pattern. *GAPDH* or 28S/18S ribosomal RNAs were used as sample loading control. (*Bottom*) Expression of *ZNF254* and *HSPC135* in hematopoietic cell lines, with *GAPDH* as control.

DISCUSSION

Because tissue- or development stage-related differential expression exists for many genes, cloning of full-length cDNA based on EST analysis in different tissues represents a useful approach for gene identification, especially for those subject to temporo-spatial regulation. In strict sense, a full-length

cDNA should cover both the ORF and the complete 5' and 3' UTR. Although a number of methods have been used to surmount the technical obstacles for getting the 5' end of cDNA (Carninci et al. 1996), it is still difficult to reach the transcription start site in many cases. However, as the most important functional information of an mRNA is contained in the ORF, cDNAs containing entire ORFs are often considered as being full-length. By combining several technologies including construction of full-length cDNA enriched libraries, in silico cloning, and RACE, a relatively efficient working system has been established to obtain full-length cDNAs, or more precisely, cDNAs including entire ORFs, in a cost-effective way. This system has enabled the first resource of cDNAs with putatively entire ORFs to be generated for previously undefined genes whose expression is found in human CD34+ HSPCs.

One strong challenge to genomic science presently is to elucidate the functions of the newly discovered huge amount of genes. In this work, we tried to apply the currently available bioinformatic tools to the analysis of the structural and functional characteristics of each ORF. Using BLAST search, 121 out of 300 ORFs were found to share homology to genes with functional information, offering important clues for the choice of appropriate functional assays in further study. The difficulty was how to deal with the majority of the ORFs without obvious functional information. We therefore attempted to evaluate the conservation of the sequences through evolution. As a result, 225 ORFs show >25% similarity at amino acid level to those identified in organisms including bacteria, *S. cerevisiae, C. elegans, Drosophila, Arabidopsis*, and nonprimate mammals, whereas 75 have so far no similarity. It is quite possible that the 21 ORFs well-conserved across a wide range of species may be derived from the "essential genes." Although a large proportion of these evolutionarily conserved genes are of unknown function, this analysis can provide at least the following information: On the one hand, they are most likely to exert important biological functions; and on the other, the lower organisms containing homologous sequences can be used as models in the functional study with gene knockout or other methods. Moreover, efforts have been made to approach the gene function by search of distinct motifs and domains with combined use of algorithms based on different methods and taking into consideration not only the primary sequence but also the secondary structure of the proteins. Of note, in addition to those well-known functional motifs such as zinc finger and leucine zipper, a putative signal peptide was found in 11 ORFs with or without transmembrane motif in proper location. This information may lead to future works to identify possible secreted proteins and transmembrane proteins, and hence may allow recognition of new regulatory pathways involved in the self-renewal and/or differentiation of HPSCs.

Characterization of gene expression with regard to tissue distribution is another way to approach the gene function. Genes with ubiquitous expression are more likely "housekeeper" genes, whereas genes whose expression shows tissue specificity may exert functions related to the development and differentiation of a given tissue or cell population. In this work, both electronic Northern and macroarray screening were carried out to study gene expression patterns. Because the majority of the genes presented in this work had been already hit by dbESTs and relevant information was available in UniGene (Boguski and Schuler 1995; Shi et al. 1999), the electronic Northern could give an approximate estimation of the tissue distribution patterns. Of note, among 270 genes thus analyzed, 207 were hit by ESTs from >10 tissues while only 13 were mainly hit by ESTs of hematopoietic tissues. On the other hand, the macroarray system with relatively high efficiency and throughput was used in this work to study gene expression

within the hematopoietic systems. Probes prepared from five hematopoietic cell lines were applied to cover granulocytic, monocytic, erythro-megakaryocytic, and lymphoid lineages. Of 285 genes expressed in CD34+ cells of cord blood origin, 35 were picked that showed relatively restricted or preferential expression along with a given orientation of differentiation. Therefore, combination of the two methods allowed us to find genes which may play a role in hematopoiesis-related functions.

In this work, we have also tried to take the opportunity of ever-increasing genomic mapping and sequence data to promote the understanding of structural organization of our genes discovered by cDNA approach. Application of bioinformatic information from public database, including sequence tag sites (STS) map (Stewart et al. 1997) and UniGene database (Boguski and Schuler 1995), allowed us to assign the chromosomal localizations for 192 novel genes. Retrieving genomic sequences from the "working draft" corresponding to our cDNAs obtained the exon-intron organizations in 243 genes, and the characterization of genomic structure of all genes can be expected in the near future with the accelerated schedule of the Human Genome Project. Although our work is only a small part in the international effort to establish a detailed whole genome transcription map, it may give some suggestions to the future study. Now, the gene discovery in genomic DNA sequencing depends largely on annotation but the successful rate based on theoretical prediction is not high enough. Hence, full-length cDNA cloning projects will provide the definitive evidence to the predicted transcription units. In contrast, genomic DNA sequences can also offer unique information for the full-length cDNA cloning. For instance, obtaining the 5' ends of genes with large coding sequence is often difficult. Exon prediction may lead experimental work to help their cloning. Besides, genes with very low expression levels or extremely narrow expression windows may be absent or poorly represented in most of the cDNA libraries. Annotation of genomic sequences may facilitate the identification of these genes. Moreover, comparison of cDNA and genomic sequences can reveal some complex mechanisms of genomic organization and expression. To this end, it is interesting to note the overlapping in reversed orientation of our HSPC070 gene and the known *RAF* gene located on chromosome 3p25, as well as the alternative splicing patterns in some genes. According to the comparative analysis between the whole genome sequence data from *C. elegans* (The C. elegans Sequencing Consortium 1998) and *Drosophila* (Adams et al. 2000), the functional complexity of a genome is determined not only by the number of the genes, but even more importantly by the alternative splicing as well as complex regulatory mechanisms of the genome at transcriptional level. Finally, the chromosomal distribution of genes bears not only evolutionary meaning, such as the mapping of all five C2H2 zinc finger genes on chromosome 19 suggestive of recent duplication events, but also indicates candidate genes in disease-related loci.

Methods

▲ TOP
▲ ABSTRACT
▲ INTRODUCTION
▲ RESULTS
▪ Methods
▼ REFERENCES

EST Sequencing and Data Analysis

Mononucleated cells were harvested from cord blood and bone marrow with gradient centrifugation and CD34+ populations were separated with anti-CD34 MAb-conjugated MACS system (Miltenyi Biotec, Germany). After two rounds of separation, CD34+ cells were of 96%-99% purity according to flow cytometry analysis (Gu et al. 2000).

RNA extraction, ZAPII cDNA libraries construction, Bluescript phagemid templates preparation, sequencing strategy, and data management were manipulated as before (Mao et al. 1998; Gu et al. 2000). The sequencing primers were universal primers including M13 Reverse and/or Forward, T3 and/orT7 primers, and sequencing mix was BigDye Terminator (Perkin Elmer). 5' or 3' end ESTs generated were categorized into known gene, dbEST, and novel EST groups by searching against GenBank database with BLAST and FASTA programs in GCG package.

Cloning of Full-Length cDNA

The EST clones corresponding to previously undefined genes were candidates for full-length cDNA cloning. The clone inserts were sequenced with end sequencing, primer extension, and sequencing after partial deletion/subcloning. AutoAssembler (Perkin Elmer) was applied to assemble the sequences into contigs. DNA Strider (Version 1.0) was employed to analyze the ORF. For those clones containing partial reading frames, in silico EST assembly and RACE were performed. Proper Marathon-ready cDNA libraries (Clontech) were chosen as RACE template, and the gene-specific primers were generated according to the clone sequence. The ORFs thus obtained were confirmed with RT-PCR.

Structure and Function Analysis with Bioinformatics

Sequence Similarity Comparison

The GCG package contains the release versions of EMBL and GenBank databases where the known genes and predicted ORFs were deposited. All amino acid sequences encoded by our cDNAs were searched against the nucleic acid sequence sub-databases of some important model organisms such as bacteria, *S. cerevisiae, C. elegans, Drosophila, Arabidopsis*, and mammals (excluding primates) with the tfasta program in the GCG package. There were two reasons to choose this strategy for homology search: First, there were many more nucleic acid sequences than amino acid sequences in the databases; second, through evolution, the amino acid sequences are more conserved than those of nucleic acid ones. In this study, two amino acid sequences were considered as homologs when they shared a similarity >25% over a region of 50-100 amino acids and the Z-score value was >200. Based on the percentages of sequence identity, these homologs were divided into 3 groups: 25%-50%, 50%-75%, and 75%-100%.

Genomic Organization Determination

The human genome sequences in GenBank (release 113) and htgs database hit by our cDNAs were retrieved, and the exon-intron organization was obtained by sequence comparison with the sim4 program (Yan et al. 1998).

Fundamental Structural and Functional Elements Searching

Programs including Motifs, Profilescan in GCG package, and Prosite at the Expacy website (http://www.expacy.ch/tools/scnpsite.html) were employed to scan for the motifs on primary structure of the peptides (Hofmann et al. 1999). Programs including Peptidestructure, Plotstructure, Pepplot, Coilscan, and Hthscan in the GCG package were applied to analyze the secondary structure of the proteins, and Spscan (GCG package) and signalP (http://www.cbs.dtu.dk/services/SignalP/), as well as

TMHMM (http://www.cbs.dtu.dk/services/TMHMM-1.0/), were used to predict the signal peptide and the α-helix transmembrane domains in those novel ORFs so as to explore the secreted or membrane anchored proteins.

Chromosomal Mapping

Electronic Mapping

dbESTs were searched to find the corresponding sequences, then UniGene database (http://www.ncbi.nlm.nih.gov/UniGene) was applied to determine the tissue expression pattern and chromosomal mapping of these novel genes (Schuler et al. 1996). The cDNA-matched genomic DNA sequence data can also provide mapping information.

Radiation Hybrid

In addition to the electronic mapping results, Stanford G3 and GeneBridge 4 Radiation Hybrid (RH) panels (Research Genetics Inc.) were applied to map the novel genes according to procedures described previously (He et al. 1998). The results were submitted to the RH Mapping Server at Stanford Human Genome Center (SHGC; http://www-shgc.stanford.edu) and Whitehead Institute/MIT Center for Genome Research (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl). SHGC or MIT framework markers linked to the subjected genes with a LOD score >6.0 were returned from the autoservers. Framework maps from SHGC, MIT, and Genethon (http://www.ceph.fr/quickmap.html) were used to infer the cytogenetic band locations corresponding to the RH mapping results.

Gene Expression in Different Tissues

In silico Northern Blot

For each entry in UniGene database (http://www.ncbi.nlm.nih.gov/ UniGene), beside the STS mapping information, cDNA source could also provide expression information.

Northern Blot

The MTN membranes used were from Clontech and the homemade membranes for hematopoietic cell lines were prepared according to the standard protocols (Sambrook et al. 1989). Probes were ^{32}P[dCTP] (DuPont) labeled with T7 quick primer (Amersham Pharmacia Biotech). Prehybridization and hybridization were performed with Expresshyb solution (Clontech). Membrane washing and autoradiography were carried out according to the standard protocol.

Screening of Gene Expression in Different Hematopoietic Cell Lines with Macroarray

Membrane Preparation

A total of 2430 unique cDNA clones corresponding to EST clusters identified in cord blood CD34+ HSPCs were PCR-amplified. The reactions were carried out using T3/T7 universal primer pairs in 50μl volume including rTaq and dNTPs (TaKaRa, Dalian, China) and on 9600 GeneAmp PCR system (Perkin

Elmer) under the following conditions: 1 min at 94°C, 1 min at 54°C, and 2 min and 20 sec at 72°C for 30 cycles and finished by an extra 10 min at 72°C. The PCR products were quantitated, precipitated with 35μl isopropanol, washed with 70% ethanol, and redissolved in 10μl 1N NaOH. BioGrid 0.4-mm 384-pins total array system (TAS) arrayer (Bio-robotics) was used to spot cDNA PCR products onto 8 × 12 cm^2 nylon membranes (Amersham Pharmacia Biotech) with duplicate spots. The cDNA samples were immobilized with UV crosslinker after drying.

Preparation of the Probes

Total RNAs were isolated with TRIzol (Life Technologies) from hematopoietic cell lines NB4, HL60, U937, K562, and Jurkat cultured under conditions described previously (Zhu et al. 1995). mRNAs were then purified from 200 μg of total RNAs with Oligotex column (Qiagen). Probes were labeled while first-strand cDNA was synthesized. A mixture containing 2 μg mRNA, 3 μl oligo(dT) primer (0.5 μg/μl), and 2 μl random primers (0.5 μg/μl) was incubated at 68°C for 5 min. Then the following items were added: 10 μl of 5× RT buffer, 1 μl of 200 mmole/l NaPP, 33 mmole dNTPs (without dATP), 15 μl [α-^{33}P]dATP (DuPont) (10 mCi/ml), 1 unit of RNase inhibitor, 60 units of AMV Reverse transcriptase (Promega), and ddH_2O to a final volume of 50 μl. The reaction was performed at 42°C for 2 hr and terminated with 100°C water bath for 5 min.

Hybridization

The spotted membranes were rinsed with 6× SSC at room temperature for 5 min, and prehybridized in 20 ml of ExpressHyb hybridization solution added with sheared salmon sperm DNA to 100 μg/μl at 68°C for 3 hr in a roller bottle. Then hybridization was carried out overnight in 5 ml of solution (ExpressHyb hybridization solution, 100 μg/μl ssDNA) mixed with the denatured cDNA probes. Washing was performed under stringent conditions (Sambrook et al. 1989): solution I (2× SSC, 0.1% SDS) at 65°C for 30 min twice and solution II (1× SSC, 0.5% SDS) at 65°C for 30 min once.

Signal Detection and Gene Expression Quantification

After stringent wash, the membranes were exposed to FLA-3000 system phosphor screens overnight, and measured with the attached ImageGauge program (Fuji). Fifteen no-sample areas were circled as background. The relative intensity for each gene was quantified after position and background correction. Only those signals with intensity value >10 could be considered as positive ones. The expression was considered as negative in the case where a negative value was recorded. The signal of housekeeping genes such as *GAPDH* or β-actin was chosen as reference for normalization, and the total signal amount of the membranes were also applied as reference. The ratio of each gene's signal to that of *GAPDH* on the same filter was chosen to compare the relative expression levels between cell lines (Pietu et al. 1999; Rhee et al. 1999).

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FOOTNOTES

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▲ TOP
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▲ INTRODUCTION
▲ RESULTS
▲ Methods
▪ REFERENCES

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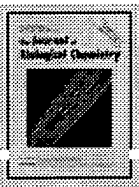

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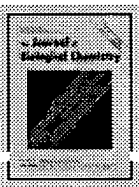

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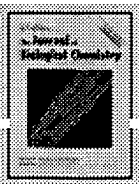

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